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Cecilia Ferrantini[1,2], MD, PhD; Josè Manuel Pioner[1], PhD; Luca Mazzoni[3], PhD; Francesca Gentile[1], PhD; Benedetta Tosi[1], MD; Alessandra Rossi[2], MD; Luiz Belardinelli[4], MD; Chiara Tesi[1], PhD; Chiara Palandri, MS[3]; Rosanna Matucci, PhD[3]; Elisabetta Cerbai[3], PhD; Iacopo Olivotto[2], MD; Corrado Poggesi[1], MD; Alessandro Mugelli[3], MD; Raffaele Coppini[3], MD, PhD.

1. Department of Experimental and Clinical Medicine, University of Florence, Italy
2. Cardiomyopathy Unit, Careggi University Hospital, Florence, Italy
3. Department NeuroFarBa, University of Florence, Italy
4. Gilead Sciences Inc, Foster City, CA, USA

Corresponding author:

Dr. Raffaele Coppini, MD, PhD

Department NeuroFarBa, division of Pharmacology, University of Florence

Viale G: Pieraccini 6, 50139 Firenze, Italy

Tel. +393400047357

e-mail: raffaele.coppini@unifi.it

Running Title:

"Ranolazine for inducible obstruction in HCM"

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ABSTRACT

BACKGROUND AND PURPOSE: In 30-40% of hypertrophic cardiomyopathy (HCM) patients, symptomatic left-ventricular (LV) outflow gradients develop only during exercise due to catecholamine-induced LV-hypercontractility (inducible obstruction). Negative-inotropic pharmacological options are limited to β-blockers or disopyramide, with scarce efficacy and tolerability.

We assessed the potential use of late Na^+-current (I_{NaL})-inhibitors to treat inducible obstruction in HCM.

EXPERIMENTAL APPROACH: The electrophysiological and mechanical responses to β-adrenergic stimulation were studied in human myocardium from HCM versus control patients. We then investigated the effects of I_{NaL}-inhibitors (ranolazine and GS-967) in HCM samples under conditions simulating rest and exercise.

KEY RESULTS: In cardiomyocytes and trabeculae from 18 surgical septal samples of patients with obstruction, the selective I_{NaL}-inhibitor GS-967 (0.5μM) hastened twitch kinetics, decreased diastolic $[Ca^{2+}]$ and shortened action potentials, matching the effects of ranolazine at a 20-times lower concentration.

The mechanical response to isoproterenol (inotropic and lusitropic) was comparable in HCM and control myocardium. However, isoproterenol prolonged action potentials in HCM myocardium, while it shortened them in controls.

At variance with disopyramide, neither GS-967 nor ranolazine reduced force at rest; however, in the presence of isoproterenol, they reduced Ca^{2+}-transient amplitude and twitch tension, while the acceleration of relaxation was maintained. I_{NaL}-inhibitors were more effective than disopyramide in reducing contractility during exercise. Finally, I_{NaL}-inhibitors abolished arrhythmias induced by isoproterenol.

CONCLUSIONS AND IMPLICATIONS: Ranolazine and GS-967 are effective in reducing septal myocardium tension during simulated exercise *in vitro* and therefore have the potential to ameliorate

symptoms caused by inducible obstruction in HCM patients, with some advantages over disopyramide and β-blockers.

KEYWORDS: hypertrophic cardiomyopathy, contractility, human myocardium, intracellular calcium, action potential, ranolazine, disopyramide, inducible obstruction, adrenergic stimulation, diastolic dysfunction

LIST OF ABBREVIATIONS:

LV=left ventricle; **LVOT**=left ventricular outflow tract; **HCM**=hypertrophic cardiomyopathy; **Dis**=disopyramide; **Ran**=ranolazine; **Iso**=isoproterenol; $\mathbf{I_{NaL}}$= late sodium current; $\mathbf{I_{CaL}}$= L-type calcium current; **EAD**= early after-depolarization; **DAD**=delayed after-depolarization; **APD**= action potential duration

INTRODUCTION

Symptoms related to obstruction occurring at the left ventricular outflow tract (LVOT) are present in approximately 65% of hypertrophic cardiomyopathy (HCM) patients (Gersh *et al.*, 2011; Authors/Task Force *et al.*, 2014). Marked upper septal hypertrophy and LV hypercontractility lead to accelerated blood flow in the LVOT, exerting a drag-force on the elongated anterior mitral valve leaflet that moves towards the septum (SAM), ultimately determining a significant pressure gradient (>30mmHg) in the LVOT (Maron *et al.*, 2006). While a significant gradient is present at rest in about half of patients with obstructive HCM , in obstruction develops only during stress or exercise in the other 50% ("dynamic" or "inducible" obstruction) (Maron *et al.*, 2006; Pozios *et al.*, 2015).

To date, pharmacological options to treat inducible obstruction are limited to beta-blockers or disopyramide. High doses of non-selective β-blockers, such as 80-100 mg/day of nadolol, are ofter

required to reduce dynamic gradients (Nistri *et al.*, 2012): a large number of patients with exertional symptoms do not tolerate such aggressive β-blocker treatments and are left with residual symptomatic gradients. Disopyramide (Dis) is an unselective compound that primarily behaves as a Na^+-channel and hERG K^+ channel blocker and is extensively used in HCM patients with rest obstruction to relieve symptoms associated with LVOT gradients or intraventricular gradients. The clinical efficacy of Dis on rest obstruction has been attributed to its negative inotropic effect: through the reduction of LV contractility, Dis slows down the acceleration of flow in the LVOT during systole, thus delaying or abolishing mitral-septal contact (Sherrid *et al.*, 2005). However, the use of Dis for inducible obstruction is limited by its unpredictable efficacy on dynamic gradients, particularly those elicited by exercise (Maron *et al.*, 2006); and by the important side effects of the drug (e.g. the anticholinergic effects). Thus, there is a strongly felt need to identify more effective and better tolerated agents for patients with obstructive HCM. However, the identification of new pharmacological options to reduce dynamic gradients is limited by the lack of studies on the cellular pathophysiology of exercise-induced obstruction. In particular, the response to β-adrenergic stimulation has never been studied in human myocardium from HCM patients.

We previously analyzed the electromechanical profile of cardiomyocytes isolated from myectomy samples of patients with obstructive HCM (Coppini *et al.*, 2013). When compared with control cells, HCM cardiomyocytes showed prolonged action potentials (APs), slower Ca^{2+}-transients and elevated diastolic Ca^{2+}, largely determined by a marked overexpression of the late Na^+ current (I_{NaL}). Such electro-mechanical abnormalities were reversed *in vitro* by the I_{NaL} inhibitor ranolazine, with beneficial effects on diastolic function and cellular arrhythmias (Coppini *et al.*, 2013). Ranolazine is available in the clinic to treat stable angina and is being employed in HCM patients with angina symptoms(Ammirati *et al.*, 2016). However, Ranolazine is a non-selective I_{NaL}-inhibitor, with several potentially relevant pleiotropic effects, such as the inhibition of K^+ and Ca^{2+} currents (Antzelevitch *et al.*, 2004), the reduction of myofilament Ca^{2+}-sensitivity (Lovelock *et al.*, 2012) and the stabilization of ryanodine receptors (Parikh *et al.*, 2012). Novel, highly selective I_{NaL} inhibitors (GS-967 and GS-6615) (Belardinelli *et al.*, 2013; Rajamani *et al.*, 2016) have been recently developed. GS-967 (6-[4-(trifluoromethoxy)phenyl]-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-

a]pyridine) is a potent inhibitor of I_{NaL}(Belardinelli *et al.*, 2013), with an IC50 of 0.2 to 0.5 μM (IC50 of ranolazine is 2-4 μM). Moreover, GS-967 has no significant effects on either peak I_{Na} or hERG K^+ current in the effective range of concentrations (Belardinelli *et al.*, 2013). GS-967 has been shown to suppress experimentally-induced ventricular arrhythmias in rat and rabbit cardiomyocytes, via shortening of AP duration and reduction of intracellular Na^+ and Ca^{2+} overload (Belardinelli *et al.*, 2013; Sicouri *et al.*, 2013; Pezhouman *et al.*, 2014); this in turn abolished the occurrence of arrhythmogenic early and delayed after-depolarizations (EADs and DADs) (Belardinelli *et al.*, 2013; Sicouri *et al.*, 2013; Pezhouman *et al.*, 2014). Moreover, GS-967, used in living porcine models, suppressed ischemia-induced and catecholamine-triggered ventricular arrhythmias (Bonatti *et al.*, 2014; Alves Bento *et al.*, 2015), as well as atrial fibrillation triggered by autonomous stimulation (Carneiro *et al.*, 2015). Notably, GS-967 did not exert any effects on the mechanical and electrical function of the rat healthy heart (Fernandes *et al.*, 2014). This result is in line with the negligible effects of ranolazine in human myocardium from non-hypertrophic non-failing patients (Coppini *et al.*, 2013), due to the absence of I_{NaL} overexpression, which appears to be a specific, disease-related, target.

In the present study we first use GS-967 as a pharmacological tool to assess whether the previously observed effects of ranolazine in human HCM myocardium can be entirely attributed to I_{NaL}-inhibition rather than its pleiotropic effects. We then compare the mechanical and electrophysiological responses of HCM myocardium to β-adrenergic agonist isoproterenol with those observed in control cardiac samples, in order to characterize the features and possible abnormalities of β-adrenergic signaling in the HCM heart. Finally, the main objective of this study is to investigate the *in vitro* effects of ranolazine and GS-967 under β-adrenergic stimulation, the latter used to simulate stress and exercise in the myocardium of patients with obstructive HCM. With this approach, we aim to assess whether the in vitro pharmacological profile of I_{NaL}-inhibitors supports their use to treat inducible obstruction in HCM patients as an alternative to disopyramide and β-blockers or in combination with these commonly used compounds.

METHODS

Details are available online ("Expanded Methods" section of the Online Data Supplement).

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

Patients: The study follows the principles of WMA Declaration of Helsinky for medical research involving human subjects. The experimental protocols were approved by the ethical committee of Careggi University-Hospital of Florence (2006/0024713, renewed May 2009; 2013/0035305). Each enrolled patient gave written informed consent. We enrolled 22 HCM patients followed by the Cardiomyopathy Unit in Florence, consecutively referred to surgical septal myectomy, for relief of drug-refractory symptoms related to LVOT obstruction. Among the 22 patients, 15 agreed to undergo mutational screening in sarcomeric genes. Clinical data are found in Table 1.

The control cohort comprised 5 patients aged <65 years undergoing heart surgery for aortic stenosis or regurgitation and who required a septal myectomy operation due to the presence of a bulging septum causing symptomatic obstruction. All control patients had septal thickness <14mm and preserved left-ventricular systolic function (ejection fraction >55%). Clinical data are found in Supplementary Table 1.

Tissue processing and cell isolation: Surgical septal specimens from HCM and control patients were washed with standard cardioplegic solution and processed within 30 minutes from excision. Endocardial trabeculae suitable for mechanical measurements were dissected and the remaining tissue was minced and subjected to enzymatic dissociation to obtain viable single myocytes, as previously described (Coppini *et al.*, 2014a).

Single cell studies: Perforated patch whole-cell current-clamp was used to measure membrane potential, as previously described (Coppini *et al.*, 2013). $[Ca^{2+}]$ variations were simultaneously monitored using the Ca^{2+}-sensitive fluorescent dye Fluoforte (Enzo Life Sciences, Farmingdale, NY), by measuring fluorescence at 515±10 nm during excitation at 490±8 nm. Cells

were mechanically permeabilized at the end of the experiment and free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from emitted fluorescence as previously described (Voigt *et al.*, 2012), using 389 nmol/L as Fluoforte dissociation constant. Late Na^+ current and L-Type Ca^{2+} current (I_{CaL}) were measured using whole-cell voltage clamp as previously described (Coppini *et al.*, 2013). I_{CaL} was also recorded under action potential clamp conditions, using representative AP traces, recorded from HCM cardiomyocytes under the same conditions, as command voltage. The inward current recorded under AP-clamp conditions was dihydropiridine-sensitive (see Supplementary Figure 6)

Intact trabeculae studies: Ventricular trabeculae were mounted between a force transducer and a motor for muscle length control, as previously described (Coppini *et al.*, 2013); isometric force was recorded under different experimental conditions and stimulation protocols. Isometric force was recorded at 35±2°C under various conditions including various pacing rates (0.1-2.5 Hz) and acute drugs administration (see below). The occurrence of premature beats was evaluated during steady state stimulation (0.5 Hz) and during stimulation pauses.

Drug studies: For experiments on isolated cardiomyocytes and trabeculae Ran was used at the concentration of 10 µM, Dis at 5 µM and GS-967 at 0.5 µM, unless otherwise specified. Each cell or trabecula was randomly assigned to be treated with one of the three test drugs (Dis, Ran or GS-967). Test recordings in presence of the drug were performed after >3 minutes from the beginning of drug exposure. Afterwards, the drug was washed out for >5 minutes and measurements were repeated. Isoproterenol (10^{-7} M) was used to mimic β-adrenergic stimulation during exercise in single cells and trabeculae.

Statistics: Raw traces were analysed by a blinded operator, different from the one who performed the experiments. For each trace, we performed measurements in at least 5 subsequent events and averaged them to obtain a single value for a given cell/trabecula in a certain condition. Clinical data from patients are expressed as mean±SD. Data from cells and muscles are expressed as mean±SEM. Figure panels 1G, 2D and 2F show the effects of drugs in a normalized fashion: we calculated the drug-induced variation of a given parameter from the baseline ([drug-baseline]/baseline, expressed as percentage) in each cell/trabecula and used these values (percentages

of variation) to calculate averages and perform statistical analyses. The number of cells/trabeculae and the number of different patient samples from which cells/trabeculae were isolated are indicated for each group/condition in the respective figure legends. For each group, we included data points from at least 5 patient samples, obtained from an even number of cells/trabeculae for each sample. Statistical analysis was performed as previously described using linear mixed models (Coppini *et al.*, 2013), taking into account non-Gaussian distribution, inequality of variances and within-subject correlation. In brief, in order to reduce the risk of type I errors resulting from the closer interrelationship between cells/trabeculae isolated from the same patient sample, we used hierarchical statistics including two nested levels (patients and cells)(Sikkel *et al.*, 2017); a third hierarchical level was added when drugs were tested in a cell/trabecula, in order to allow paired comparisons. This approach was implemented using linear mixed models in Stata 12.0 (StataCorp LLC, USA). For categorical data (e.g. occurrence of cellular arrhythmias), we used the Fisher exact test. $P<0.05$ was considered statistically significant. Due to poor availability of control samples, the number of control cells/trabeculae included in the unpaired comparisons (Fig.2) is lower than that of HCM cells/trabeculae.

RESULTS

GS-967 shortens action potentials and accelerates the kinetics of Ca^{2+} transients and twitches in HCM cardiomyocytes.

GS-967 was tested at 0.5 µM in isolated cardiomyocytes from HCM patients during patch-clamp and Ca^{2+}-fluorescence measurements (Fig 1A). GS-967 consistently reduced I_{NaL} by 66±8% on average (Fig 1A). Moreover, GS-967 shortened the duration of action potentials at all frequencies studied (Fig. 1B-C). Average reduction of APD90% was 23.3±7.4 % at 0.2 Hz, 22.3±6.2 at 0.5Hz and 21.4±6.7 % at 1 Hz. Moreover, GS-967 reduced the rate of arrhythmogenic early-afterdepolarizations (EADs) occurring spontaneously during regular stimulation for 3 minutes. The number of cells showing EADs during 3 minutes of stimulation was halved by GS-967 (Supplementary Fig. 2). Notably, GS-967 had no effects on the upstroke time nor on the amplitude of the action potentials (Supplementary Table 3). The kinetics of intracellular Ca^{2+} transients was hastened by GS-967 at all

frequencies investigated (Fig. 1D-E): both the rise and decay times of Ca^{2+} transients were shortened by GS-967 application (Supplementary Table 3). GS-967 reduced diastolic [Ca^{2+}], while the amplitude of Ca^{2+} transients was unaffected (Fig. 2F-G). Accordingly, GS-967 reduced the occurrence of delayed after-depolarisations (Supplementary Fig. 2). GS-967 was then tested in intact trabeculae from HCM patients, while recording force under isometric conditions (Fig. 2H) during stimulation at different frequencies (0.1 Hz to 3 Hz). GS-967 shortened twitch duration mainly by reducing time to peak contraction (Fig. 2J). GS-967 (0.5μM) had no effects on action potentials and Ca^{2+} transients recorded from cardiomyocytes isolated from control patients (Supplementary Figure 3).

B-adrenergic stimulation exerts comparable mechanical effects in HCM and control myocardium, but causes a paradoxical prolongation of action potentials in HCM cardiomyocytes.

The effects of β-adrenergic stimulation with isoproterenol (10^{-7} M, Iso) on isometric twitches were tested in trabeculae from control and HCM septal samples (Fig. 2 A-D). Iso exerted qualitatively and quantitatively similar effects on twitch amplitude and kinetics in control and HCM trabeculae. In particular, Iso increased peak force by 3.4±0.6 times in control and 3.2±0.5 times in HCM trabeculae (Fig. 2 B). Twitch kinetics was slower in HCM vs. control trabeculae both at baseline and under β-adrenergic stimulation (Fig. 2 C). Indeed, Iso accelerated the kinetics of relaxation by a similar amount in HCM and control trabeculae; however, the acceleration of twitch force development was less pronounced in HCM trabeculae (Fig. 2 D). All in all, the kinetics of contraction and relaxation, despite being accelerated by isoproterenol, was still slower in HCM vs. control trabeculae even during maximal β-adrenergic stimulation.

Contrarily, the effects of Iso on action potentials were profoundly different in HCM with respect to control cardiomyocytes (Fig. 2 E). While Iso shortened the duration of action potentials in control cardiomyocytes, β-adrenergic stimulation prolonged repolarization in all cardiomyocytes from HCM patients (Fig. 2 F).

Ranolazine and GS-967 decrease isometric force under β-adrenergic stimulation.

We tested the effects of Dis (5 μM), Ran (10 μM), GS-967 (0.5 μM) and propranolol (0.1 μM) on isometric twitch amplitude and kinetics in trabeculae from HCM patient samples, in the absence and presence of β-adrenergic stimulation with isoproterenol (10^{-7} M, Iso), used to simulate exercise or stress conditions. Under baseline conditions, Dis reduced twitch amplitude by approximately 50%, while force was unaffected by Ran, GS-967 and propranolol (Fig.3A&C). β-adrenergic activation with Iso led to the expected increase of twitch amplitude and hastening of twitch kinetics (Fig. 3A,B,D). Interestingly, Dis, Ran, GS-967 and propranolol reduced twitch amplitude when applied on top of isoproterenol (Fig. 3A-C). The reduction of twitch amplitude under Iso was quantitatively less pronounced with Dis (-16±7%) as compared with Ran (-56±23%, $P< 0.01$ vs. Dis) or GS-967 (-32±21%, $P< 0.05$ vs. Dis; see Fig. 3A&C). The reduction of twitch force in the presence of Ran or GS-967 was comparable with that obtained by blocking β-adrenergic receptors with propranolol (Fig.3C). Nonetheless, the acceleration of twitch kinetics by Iso was not antagonized by the application of Dis, Ran or GS-967 (Fig. 3A,B,D). Indeed, after the addition of these drugs on top of Iso, both time to peak and 50% relaxation time remained as fast as in the presence of Iso alone (Fig.3D). Contrarily, propranolol completely antagonized the lusitropic effect of isoproterenol (Fig.3D).

Cellular mechanisms underlying the effects of I_{NaL}-inhibitors under isoproterenol.

Ran and GS-967 were similarly tested in isolated HCM cardiomyocytes (Fig.4, Table 2). In line with the effects observed on twitch amplitude in trabeculae, Ran and GS-967 did not affect Ca^{2+} transient amplitude at baseline (Fig.4A). Ca^{2+} transient amplitude increased in response to Iso by 14±3%. The application of either Ran or GS-967 on top of Iso significantly reduced Ca^{2+} transient amplitude (Fig. 4B). Interestingly, while Iso did not affect intracellular diastolic [Ca^{2+}], Ran and GS-967, applied on top of Iso, significantly reduced diastolic [Ca^{2+}] (Fig. 4C). Interestingly, Iso

accelerated Ca^{2+}-transient decay but prolonged Ca^{2+}-transient rise time in HCM cardiomyocytes (Fig. 4A, Table 2). Accordingly, Iso slowed time-to-peak shortening in HCM cardiomyocytes, but accelerated relaxation (Supplementary Fig.1). Ran and GS-967 shortened Ca^{2+} transient rise when applied on top of β-adrenergic stimulation with Iso, but did not affect Ca^{2+} transient decay kinetics (Fig. 4A, Table 2). In line with that, Ran and GS-967 reverted the prolongation of action potentials induced by Iso (Fig 4 A&D). Contrarily, ranolazine (on top of isoproterenol) did not affect the duration of APs in control myocytes (Supplementary Figure 4A-B); in line with that, GS-967 did not reduce twitch force during β-adrenergic stimulation in control trabeculae (Supplementary Figure 4C-D). We excluded that the reduction of Ca^{2+} transients and force by I_{NaL}-inhibitors under β-stimulation depends on a partial block of β-adrenergic receptors by these compounds (Supplementary Figure 5): binding studies showed that ranolazine has a very mild interaction with β1 and β2 receptors at the concentration used in this work (10μM), while GS-967 does not bind β-receptors, even at very high concentrations. In further support of this hypothesis, we found that ranolazine exerted a negative inotropic effect also when the positive inotropic effect of β-adrenergic stimulation was mimicked by forskolin, thereby bypassing the β-receptors (Supplementary Figure 4E-F). Finally, we investigated whether inhibition of L-Type Ca^{2+} current by I_{NaL}-inhibitors contributed to the observed effects (Fig. 5). We found that neither ranolazine nor GS-967 affect the peak density of I_{CaL} in HCM cardiomyocytes (Fig. 5A-B). Interestingly, we noticed that isoproterenol not only increases the amplitude of I_{CaL}, but also markedly slows down current's inactivation in HCM cells (Fig. 5C-D). Using the AP-clamp technique, we found that the prolongation of action potential by Iso increases the total inward flow of Ca^{2+} during the plateau of the AP (Fig.5E-F): this is mainly determined by the direct effects of β-stimulation on I_{CaL} (i.e.the increased I_{CaL} amplitude and prolonged inactivation) rather than being a consequence of AP prolongation (Supplementary Fig.6). Ranolazine,added on top of Iso, markedly reduces the total inward flow of Ca^{2+} in HCM myocytes (Fig. 5E-F): this is a consequence of the marked shortening of action potentials caused by I_{NaL} inhibition under β-adrenergic stimulation (Supplementary Fig.6).

I_{NaL}-inhibitors abolish cathecolamine-induced arrhythmia in HCM myocardium.

We then evaluated the effects of Ran and GS-967 on cellular arrhythmias evoked by β-adrenergic stimulation (Fig.6A-B). Iso markedly increase the occurrence of both early and delayed after-depolarisations in HCM cardiomyocytes (Fig.6). Interestingly, both Ran (10μM) and GS-967 (0.5 μM) reduced the occurrence of EADs and DADs.

Finally, we evaluated the frequency of premature spontaneous beats and sustained triggered activity occurring in HCM trabeculae during stimulation pauses in the presence of Iso (Fig. 7 A&B). When added on top of Iso, Ran and GS-967 markedly reduced the occurrence of arrhythmic events (Fig. 7 C), in keeping with the reduction of EADs and DADs observed in isolated HCM cardiomyocytes.

DISCUSSION

Selective I_{NaL} inhibition: a disease-specific pharmacological option for HCM treatment

I_{NaL} is three times larger in the myocardium of HCM patients compared to that of non-failing non-hypertrophic subjects, leading to a prolonged action potential duration that we observed consistently in myocardial samples from over 30 HCM myectomy patients (Coppini *et al.*, 2013). In our view, these results highlight I_{NaL} inhibition as a disease-specific pharmacological strategy to treat HCM. We previously confirmed this hypothesis by acutely applying ranolazine on myocardial samples form myectomy HCM patients, where we observed marked beneficial effects on diastolic function and arrhythmogeneicity (Coppini *et al.*, 2013). In this work, a novel, more selective I_{NaL} inhibitor, GS-967, was tested in intact cardiomyocytes and trabeculae from 18 HCM patients, in order to confirm that the beneficial effects previously observed with ranolazine (Coppini *et al.*, 2013) were indeed consequence of I_{NaL} inhibition and were not mediated by the other pleiotropic effects of the drug (such as inhibition of K^+ and Ca^{2+} currents (Antzelevitch *et al.*, 2004), reduction of myofilament Ca^{2+}-sensitivity (Lovelock *et al.*, 2012), stabilization of ryanodine receptors (Parikh *et al.*, 2012) or a

mild beta-blocker effect (Flenner *et al.*, 2016)). Here, we found that all the effects of GS-967 observed in HCM myocardium are qualitatively and quantitatively similar to those previously obtained with ranolazine, but they are achieved at a 20-times lower concentration (0.5μM GS-967 vs. 10μM ranolazine), owing to the increased potency and selectivity for I_{NaL}. As an example, 0.5μM GS-967 reduced I_{NaL} in HCM myocytes by 67% (Fig.1), while ranolazine inhibited 72% of I_{NaL} at 10μM (Coppini *et al.*, 2013). Moreover, GS-967, just like ranolazine, shortened AP duration, reduced the risk of arrhythmogenic EADs (Fig.1), lowered diastolic $[Ca^{2+}]$, suppressed the risk of DADs, accelerated Ca^{2+} transient kinetics and hastened twitch relaxation in HCM myocardium (Fig.1). All in all, our results indicate that GS-967 may exert the same possible beneficial effects as ranolazine on arrhythmias and diastolic function, the main determinants of symptoms and outcome in HCM patients (Coppini *et al.*, 2014b; Olivotto *et al.*, 2015).

These results also have important mechanistic implications. Since GS-967 does not affect any other relevant target besides I_{NaL} at this concentration (Belardinelli *et al.*, 2013), our results confirms that the reduction of arrhythmogeneicity and the amelioration of diastolic function, previously observed with ranolazine (Coppini *et al.*, 2013), are a direct consequence of I_{NaL} inhibition. Of note, we observed no major effects of GS-967 in cardiomyocytes from control patients (Supplementary Fig. 3), as previously observed with ranolazine (Coppini *et al.*, 2013), suggesting that the efficacy of I_{NaL} inhibitors is specific to disease conditions where a pathological increase of I_{NaL} is observed.

In addition to the acute effects on myocardial function, I_{NaL}-inhibition may lead to additional benefits during long-term treatment. Work from our group showed that lifelong I_{NaL} inhibition in a mouse model of HCM prevents the development of hypertrophic phenotype and LV dysfunction(Coppini *et al.*, 2017) and suggest that I_{NaL} inhibition with ranolazine or more selective agents may be an effective disease-modifying strategy in patients with HCM.

I_{NaL} inhibitors: novel options to treat inducible obstruction?

The principal aim of this work is to investigate the potential of I_{NaL} inhibitors in HCM further from the two previously evidenced rationales, i.e. reduction of the arrhythmic risk and the amelioration of symptoms related to diastolic dysfunction. Here we tested these compounds as negative-inotropic

agents to decrease exercise-induced LV septal hypercontractility, which is considered a direct cause of obstruction-related symptoms in patients with obstructive HCM.

The negative-inotropic agents that are currently used to treat obstructive HCM are limited, particularly for the cases of inducible obstruction. Despite its widespread use, disopyramide is far from being the perfect drug to control obstructive symptoms: it has been successfully employed for more than 40 years to treat rest obstruction but has a very limited efficacy on exercise-induced LVOT gradients (Maron *et al.*, 2006). In addition, anticholinergic undesired effects (mouth dryness, constipation, drowsiness) are relatively common and may require discontinuation of the drug unless they may be controlled by pyridostigmine (Sherrid, 2016). β-blockers, the only available therapeutic option to treat inducible obstruction, are often insufficient to control symptoms, and the high dosages required to significantly impact on dynamic LVOT gradients may be poorly tolerated (Nistri *et al.*, 2012).

Our results show that selective I_{NaL}-inhibitors reduce myocardial force only during intense β-adrenergic stimulation, mimicking stress or exercise, while leaving baseline ("rest") contractility unaffected. Interestingly, the lusitropic effect of β-adrenergic stimulation (i.e. acceleration of relaxation) is maintained, while only the positive inotropic effect is lost. This is at variance with β-blockers which, by acting at receptor level, antagonize the full spectrum of β-adrenergic effects, including the acceleration of relaxation (Fig.3) and the increase in heart rate, both essential for tolerating intense exercise activity. Interestingly, this effect appears to be specific of HCM myocardium, as the reduction of contractile force by I_{NaL} inhibitors during adrenergic stimulation is not observed in myocardial samples from control patients (Supplementary Fig. 4). The cellular mechanisms underlying the unexpected negative inotropic action of I_{NaL} inhibitors under β-adrenergic stimulation in HCM myocardium have been extensively investigated.

Mechanisms underlying the negative-inotropic action of I_{NaL}-inhibitors under β-adrenergic stimulation in HCM myocardium.

1)Altered electrical response to β-adrenergic stimulation in HCM myocardium

By directly comparing the response of HCM myocardium to β-adrenergic stimulation with that of control cardiac muscle (Fig. 2), we here provided the first characterization of the features and abnormalities of β-adrenergic signaling in human hearts from HCM patients. In particular, we observed that the mechanical response to isoproterenol in terms of increased force amplitude and relaxation velocity was comparable in HCM and control trabeculae, but contractile kinetics remains slower in HCM vs. control trabeculae, even during maximal β-adrenergic stimulation. These results indicate that the subcellular mechanisms responsible for the lusitropic effects of β-adrenergic cascade are essentially preserved in HCM myocardium: HCM myofilaments retain the ability to decrease Ca^{2+}-sensitivity upon phosphorylation of troponin-I by PKA (Sequeira *et al.*, 2013), and PKA-mediated phosphorylation of phospholamban occurs normally in human HCM myocardium (Coppini *et al.*, 2013; Helms *et al.*, 2016).

On the contrary, the electrophysiological response to isoproterenol in isolated HCM cardiomyocytes was profoundly abnormal (Fig 2E-F). Among the many different effects on sarcolemmal and SR targets, Iso enhances both L-Type Ca^{2+} current and the slow delayed rectifier K^+ current (I_{Ks}), with the latter prevailing in healthy human cells (Terrenoire *et al.*, 2005), thus leading to a net reduction of the APD (Taggart *et al.*, 2003) in control myocardium (Fig. 2E-F). In HCM cardiomyocytes, hypertrophic remodeling leads to the unbalanced changes in the expression of Ca^{2+} and K^+ currents (Coppini *et al.*, 2013), that is, the expression of all K^+ channels (including those responsible for I_{Ks}) is greatly reduced in the presence of a normal (or even slightly increased) density of I_{CaL}, as compared with control myocardium. In HCM myocytes, in response to β-stimulation, the potentiation of I_{CaL} prevails over the increase of K^+ currents, thus leading to a net increase of depolarizing currents during the plateau of the AP, causing the observed "paradoxical" prolongation of APD (Fig. 2E-F). Moreover, in HCM cardiomyocytes, β-adrenergic stimulation not only increases peak I_{CaL} amplitude, but also markedly slows down I_{CaL} inactivation (Fig. 5). The marked slowing of I_{CaL} inactivation is likely to contribute to the prolongation of APs by β-adrenergic stimulation. Additionally, it was previously shown that beta-adrenergic stimulation of ventricular cardiomyocytes with isoproterenol leads to I_{NaL} enhancement (Dybkova *et al.*, 2014) through activation of CaMKII. A further increase of I_{NaL} following β-adrenergic stimulation in HCM myocytes may have contributed to

the paradoxical prolongation of APs observed in these cells. This paradoxical response might be relevant for the pathophysiology of exercise or stress-induced arrhythmias in HCM patients, as AP prolongation increased the risk of arrhythmogenic early-afterdepolarizations and triggered activity in HCM myocardium (Fig. 5 and 6).

2)Altered calcium response to β-adrenergic stimulation in HCM myocardium

In HCM cardiomyocytes, unlike control myocardium, the β-adrenergic-dependent increase of Ca^{2+} release and force may greatly rely on the increase in the duration of Ca^{2+} entry via L-Type Ca^{2+} channels caused by by the slower I_{CaL} inactivation). In addition to the enhancement of I_{CaL}, increased Ca^{2+}-entry via reverse-mode NCX contributes to augment Ca^{2+}-transients upon β-stimulation (Perchenet *et al.*, 2000); the latter is likely to be a consequence of the elevation of intracellular $[Na^+]$ in response to the enhancement of I_{NaL} and the prolongation of AP plateau that follows β-adrenergic stimulation (Dybkova *et al.*, 2014) (Coppini *et al.*, 2013). Indeed, we found that the rise time of Ca^{2+} transients prolongs in response to Iso in HCM cardiomyocytes (Fig. 4, Table 2). The prolongation of Ca^{2+}-transient rise time may have direct mechanical consequences: indeed, we observed that the acceleration of twitches' rising phase (time to peak) upon β-stimulation was significantly less pronounced in HCM vs. control trabeculae (Fig. 2D). The idea that the increase of force in response to β-adrenergic stimulation mainly depends on the increase of sarcolemmal Ca^{2+}-entry is in apparent contrast with the commonly accepted idea that the positive inotropic effects of β-stimulus stem from the increase of sarcoplasmic reticulum Ca^{2+} content (Desantiago *et al.*, 2008), due to enhancement of SERCA function by PKA-mediated phospholamban phosphorylation. The observed acceleration of Ca-transient decay under Iso in HCM cardiomyocytes (Fig.4 and Table 2) suggests that the β-adrenergic-induced increase of SERCA function is preserved in HCM myocardium. The increase of SR Ca^{2+} content following β-stimulation may be limited by the increased diastolic leakage from hyper-phosphorylated ryanodine receptors (due to increased Calmodulin-kinase activity, see Coppini *et al.*, 2013 and Ferrantini *et al* 2016)., thus rendering inotropic responses more dependent on the increase of Ca^{2+} entry through the sarcolemma. Another possible contributor to this phenomenon is the reduced density of t-tubules (Orchard *et al.*, 2008). We performed a preliminary investigation of

the density of T-tubules in cells isolated from the 10 HCM patient samples included in the study (Supplementary Fig. 7): in all the cells observed, T-tubule density was extremely low, much lower than the expected for healthy myocardium (Lyon *et al.*, 2009). The functional consequences of β-adrenergic activation may be radically different in disease myocytes with a markedly reduced density of T-tubules. In the near-absence of t-tubules and with a large redistribution of L-Type Ca^{2+}-channels to the surface sarcolemma (Coppini *et al.*, 2013), modulation of myocardial inotropism becomes largely dependent on the amplitude of Ca^{2+} trigger (Ferrantini *et al.*, 2014), that is, the density and duration of I_{CaL} plus the rate of NCX-mediated Ca^{2+} entry (reverse mode). Due to the reduced density of t-tubules in HCM cardiomyocytes, the prolongation of APs is likely to be essential for the positive-inotropic effect of β-adrenergic activation. All in all, the abnormal Ca^{2+} response to β-stimulus may contribute to impair relaxation during exercise, leading to exercise intolerance and exertional symptoms in HCM patients, regardless of obstruction.

3) Effects of I_{NaL}-inhibitors on APs under β-adrenergic stimulation.

The abnormal response of HCM myocardium to β-stimulation in terms of AP duration and Ca^{2+} handling underlies the unexpected negative inotropic action of I_{NaL}-inhibitors. We found that both ranolazine and GS-967, when applied on top of Iso, cause shortening of AP and Ca^{2+} transient duration and reduce Ca^{2+} transient amplitude (Fig. 4). On the contrary, AP duration does not change in response to ranolazine in control cardiomyocytes under Isoproterenol (Supplementary Figure 4). Interestingly, AP shortening by I_{NaL}-inhibitors occurs also in the absence of β-stimulation (see Figure 1 for GS-967 and Coppini *et al.* 2013 for ranolazine). However, when we compare the degree of APD shortening by I_{NaL}-inhibitors at baseline (in a total of >50 cells combined) with that obtained under Iso (in a total of >30 cells), the effect is significantly more pronounced in the presence of β-stimulation: at 0.5Hz pacing rate, reduction of APD was -27.1±3.7% in the presence of Iso, while it was -17.2±2.4% at baseline (P<0.05, linear mixed models). When APD is prolonged and repolarizing K^+ currents are reduced, the duration of AP plateau is more dependent on I_{NaL} (Wu *et al.*, 2011; Shattock *et al.*, 2017): therefore, the reduction of APD following a proportionally comparable inhibition of I_{NaL} is larger when APD is longer and I_{NaL} is further increased (i.e. in the presence of isoproterenol). The

prolongation of APD in response to isoproterenol leads to an increase in the frequency of early after-depolarizations (EADs) in HCM cardiomyocytes. Interestingly, when I_{NaL}-inhibitors are added on top of β-stimulation, the shortening of APD brings the rate of EADs back to baseline, suggesting a possible efficacy of I_{NaL}-inhibitors in preventing stress-induced arrhythmias in HCM.

4) Effects of I_{NaL}-inhibitors on Ca^{2+}-transients and diastolic [Ca^{2+}] under β-adrenergic stimulation.

I_{NaL} inhibitors, via shortening of APD, antagonize the increase of the duration of I_{CaL} during the plateau of APs induced by Iso in HCM cardiomyocytes, as demonstrated here by AP-Clamp experiments (Figure 5 and Supplementary Fig.6), ultimately leading to a marked reduction of total Ca^{2+} entry via I_{CaL}(-65±12% with respect to Iso). This effect is a direct consequence of the shortening of APs and does not depend by any direct interactions of I_{NaL} inhibitors with Ca^{2+} channels (Figure 5). Moreover, in the presence of β-stimulation, I_{NaL} inhibition with ranolazine may reduce intracellular [Na^{+}] by a greater extent as compared with the "rest" condition. The marked reduction in intracellular [Na^{+}] due to I_{NaL} inhibition (Coppini *et al.*, 2013; Kornyeyev *et al.*, 2015) during β-stimulation may lead to a pronounced decrease in reverse-mode NCX activity, which in turn may contribute to diminish Ca^{2+} entry during AP plateau.

In line with the reduction of Ca^{2+} entry via I_{CaL} and reverse-mode NCX, time-to peak Ca^{2+}-transients (markedly prolonged by isoproterenol) is brought back to baseline levels by the addition of I_{NaL} inhibitors (Figure 4 and Table 2). The reduction of isoproterenol-induced AP prolongation by I_{NaL}-inhibition combined with the decrease of [Na^{+}] may prevent the increase of Ca^{2+} entry through I_{CaL} and reverse-mode NCX contractility by β-stimulation in HCM myocardium. The marked reduction of Ca^{2+} entry appears to be large enough to counteract the isoproterenol-induced increase of Ca^{2+} transient amplitude and force in HCM myocardium, while I_{NaL}-inhibitors are unable to significantly reduce Ca^{2+}-transient and twitch amplitude in the absence of Iso (Fig.1,3 and 4). In parallel with the decrease of reverse-mode NCX, the marked reduction of [Na^{+}] by I_{NaL}-inhibition also leads to increased forward activity of the exchanger (Coppini *et al.* 2013), thus increasing the rate of Ca^{2+} efflux through the sarcolemma during the diastolic period. This may have directly contributed to

the marked reduction of diastolic [Ca^{2+}] observed when ranolazine or GS-967 are added on top of isoproterenol (Fig. 4A-4C). The increased sarcolemmal efflux combined with the decreased Ca^{2+} influx through I_{CaL} is likely to cause a reduction of SR Ca^{2+} content (Trafford *et al.*, 2001; Eisner *et al.*, 2013) in response to I_{NaL}-inhibition under Iso. A decrease of SR Ca^{2+} load may reduce diastolic leakage, contributing to the decrease of diastolic cytosolic [Ca^{2+}] (Ferrantini *et al.*, 2016). In line with that, the frequency of diastolic Ca^{2+} waves leading to delayed after-depolarizations (markedly increased by isoproterenol) is reduced by I_{NaL}-inhibitors in HCM cardiomyocytes under β-stimulation.

The weak β-blocker action of ranolazine is not responsible for its negative inotropic effect under isoproterenol.

Ranolazine was previously shown to behave as a weak β-adrenoceptor blocker (Letienne *et al.*, 2001; Flenner *et al.*, 2016), albeit at higher concentrations than that used in our experiments (20μM or higher). We here confirmed that ranolazine mildly binds to β1 and β2 adrenergic receptors at the concentration used in our experiments (10μM); on the contrary, GS-967 does not interact with β-receptors, even at high concentrations (Supplementary Figure 5). The mild β-blocking effect of ranolazine is unlikely to play a major role in the observed response of HCM muscle to ranolazine under Iso, for the following reasons: (I)The acceleration of relaxation by Iso is not antagonized by 10μM ranolazine, only the increase in force amplitude is reduced. (II) The same negative inotropic effect as ranolazine is observed with 0.5μM GS-967 (Fig 3E), which does not have any β-blocking capabilities (Supplementary Figure 5), in line with previous observations in different healthy and diseased animal models (Belardinelli *et al.*, 2013; Fernandes *et al.*, 2014; Alves Bento *et al.*, 2015). (III) Finally, when we mimicked the inotropic and lusitropic response to β-stimulation with forskolin in HCM trabeculae (thereby bypassing β-receptors), ranolazine, added on top of forskolin, exerted the same negative inotropic effect that was observed in the presence of β-stimulation with isoproterenol (Supplementary Figure 4). These observations confirm that the interaction of I_{NaL} inhibitors with the physiological consequences of β-stimulation occurs downstream of the β-receptor and of adenylyl cyclase activation and is a sole consequence of the inhibition of I_{NaL}.

Clinical Implications and conclusions

In HCM myocardium, disopyramide exerts a potent negative inotropic effect at rest but is less effective under β-adrenergic stimulation (Figure 3). This provides a clear explanation of why disopyramide effectively reduces resting gradients but is inadequate to control symptoms in patients with inducible obstruction. Conversely, I_{NaL}-inhibitors (ranolazine and GS-967) reduce myocardial force only during β-adrenergic stimulation, used here to simulate exercise and stress. Thanks to this effect, ranolazine and selective I_{NaL} inhibitors(Justo *et al.*, 2016; Rajamani *et al.*, 2016) may be effective in reducing septal tension during exercise and therefore have a theoretical potential to ameliorate symptoms caused by inducible obstruction in HCM. Furthermore, by limiting Ca^{2+} overload during stress, I_{NaL} inhibitors may also play a protective role against stress-induced arrhythmias[25, 26]. Intriguingly, both ranolazine and GS-967 abolished the increase in spontaneous contractions and triggered activity induced by isoproterenol (Fig. 5 and 6). By acting downstream of the receptor, I_{NaL} inhibitors only counteract the positive inotropic effect of β-adrenergic stimulation on septal tension and do not reduce the lusitropic and the chronotropic effects of norepinephrine, which are essential to tolerate exercise. High doses of non-selective β-blockers, such as nadolol, are often required to reduce dynamic gradients in patients with exertional symptoms due to inducible obstruction (Nistri *et al.*, 2012): a large number of patients do not tolerate such aggressive β-blocker treatments and are left with residual symptomatic gradients. In other patients, residual dynamic gradients persist even at the maximal target dose of β-blockers (e.g. 80-100 mg/day of nadolol, see (Nistri *et al.*, 2012). Based on our results, we can envision the use of I_{NaL} inhibitors in addition to β-blockers in patients with residual obstruction-related symptoms at the maximal tolerated dose of β-blockers, with the purpose of abolishing the remaining inducible gradients.

In conclusion, the novel selective I_{NaL}-inhibitor GS-967 improved diastolic function and reduced the arrhythmogenic potential of HCM myocardium in vitro, matching the effects of ranolazine at 20-times lower concentrations. I_{NaL}-inhibitors (ranolazine and GS-967) appeared to reduce septal contractility at peak exercise without affecting resting performance. These data suggest improved efficacy of I_{NaL}-inhibitors over disopyramide, and a synergistic action to β-blockers, in HCM patients with exercise-induced obstruction.

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Author Contributions: Dr. Ferrantini devised the project, performed intact trabeculae experiments, analysed the data and wrote the manuscript. Dr. Pioner contributed to intact trabeculae experiments and to analyse the data. Dr. Mazzoni collected and processed cardiac samples and contributed to single cell experiments. Dr. Gentile and Dr. Tosi contributed to intact trabeculae experiments. Dr. Rossi collected clinical data from patients. Dr. Belardinelli contributed to devise the project and critically reviewed the

manuscript. Dr. Matucci performed β-receptors binding experiments. Dr. Palandri contributed to single cell experiments. Prof. Tesi and Prof. Cerbai contributed to data analysis and interpretation and edited the manuscript. Dr. Olivotto recruited HCM patients and reviewed the manuscript. Prof. Poggesi and Prof. Mugelli contributed to conceive the project and critically edited the manuscript. Dr. Coppini devised the experiments, performed single cell experiments, analysed and interpreted the results and wrote the manuscript.

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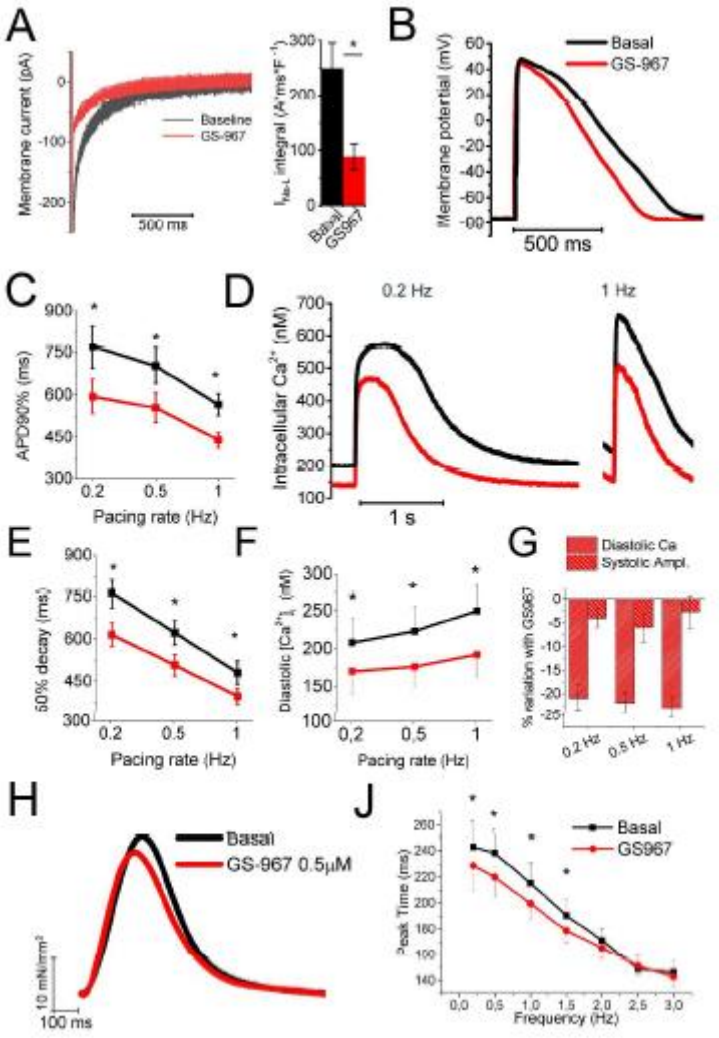

Figure 1: (A) Left: representative I_{NaL} traces from an HCM cardiomyocyte during depolarization to -20mV in the absence (Basal) or presence of GS-967 (0.5 µmol/L). Right: Average integrals of I_{NaL} calculated between 50 and 750 ms from the onset of depolarization; Means±SE from 14 HCM myocytes (5 patients). **(B)** Representative superimposed action potentials at baseline (black trace) and in the presence of GS-967 0.5µM (red trace), elicited at 0.2 Hz. **(C)** Action potential duration at 90% of repolarization (APD90%) at baseline (black) and in the presence of GS-967 0.5µM (red). Means ± standard error from 37 HCM cardiomyocytes from 9 HCM patients. **(D)** Representative superimposed Ca-transients at baseline (black traces) and in the presence of GS-967 0.5µM (red traces), elicited at 0.2 Hz (left) and 1 Hz (right). **(E)** Time from peak to 50% decay of Ca-transients (50% Decay) at baseline (black) and in the presence of GS-967 0.5µM (red), elicited at 0.2, 0.5 and 1 Hz. **(F)** Diastolic

intracellular Ca-concentration ($[Ca^{2+}]_i$) at baseline (black) and in the presence of GS-967 0.5µM (red), during regular stimulation at 0.2, 0.5 and 1Hz at steady state. **(G)** % variation of diastolic $[Ca^{2+}]_i$, and Ca-transient amplitude (Systolic Ampl.) with the application of GS-967 with respect to baseline in HCM cardiomyocytes. **(E-G)** Means ± standard error from 26 cardiomyocytes from 7 HCM patients. **(H)** Representative superimposed force twitches elicited at 0.5Hz in HCM trabeculae in the absence (black trace) and presence (red trace) of GS-967. **(J)** Time from stimulus to peak of force twitches elicited at different frequencies (0.1 to 3 Hz) at baseline (black), in the presence of GS-967 (red) and after 10 minutes from drug washout (grey). Means ± standard error from 10 trabeculae from 7 patients. *=$P<0.05$, linear mixed models corrected for paired comparisons.

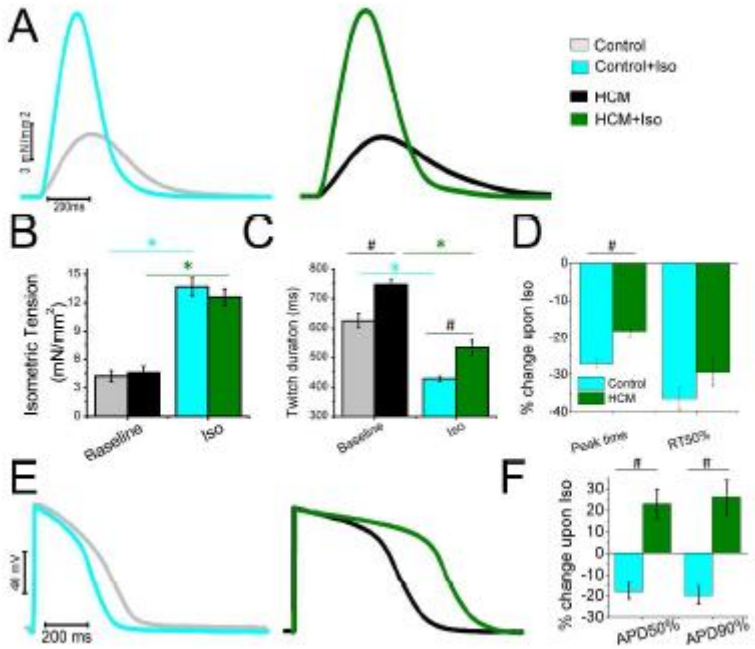

Figure 2: Mechanical and electrical response to β-adrenergic stimulation of HCM myocardium. **(A)** Representative superimposed force twitches elicited at 0.5Hz in control (left) and HCM (right) trabeculae in the absence and presence of Isoproterenol 10^{-7}M (Iso). **(B)** Isometric tension during steady state stimulation at 0.5 in the absence and presence of Iso in control and HCM trabeculae **(C)** Duration of force twitches (from stimulus to 90% of relaxation) elicited at 0.5 Hz in the absence and presence of Isoproterenol 10^{-7}M **(D)** Percentages of Change in the parameters of twitch kinetics (0.5 Hz) upon exposure to Iso in Control (cyan) and HCM (green) trabeculae: time from stimulus to peak contraction (peak time) and time from peak to 50% of relaxation (RT50%). **(B-D)** Means ± standard error from 6 trabeculae from 5 control patients and 30 trabeculae from 20 HCM patients. **(E)** Representative superimposed action potentials elicited at 0.5 Hz in control (left) and HCM (right) cardiomyocytes, in the absence and presence of Iso. **(F)** Percentages of Change in the parameters of action potential kinetics upon exposure to Iso in Control (cyan) and HCM (green) cardiomyocytes: time from stimulus to 50% repolarization (APD50%) and time from peak to 90% of repolarization (APD90%) **(F)** Means ± standard error from 13 cardiomyocytes from 5 control patients and 31 cardiomyocytes from 9 HCM patients. #=P<0.05, linear mixed models, unpaired comparisons; *=P<0.05, linear mixed models corrected for paired comparisons.

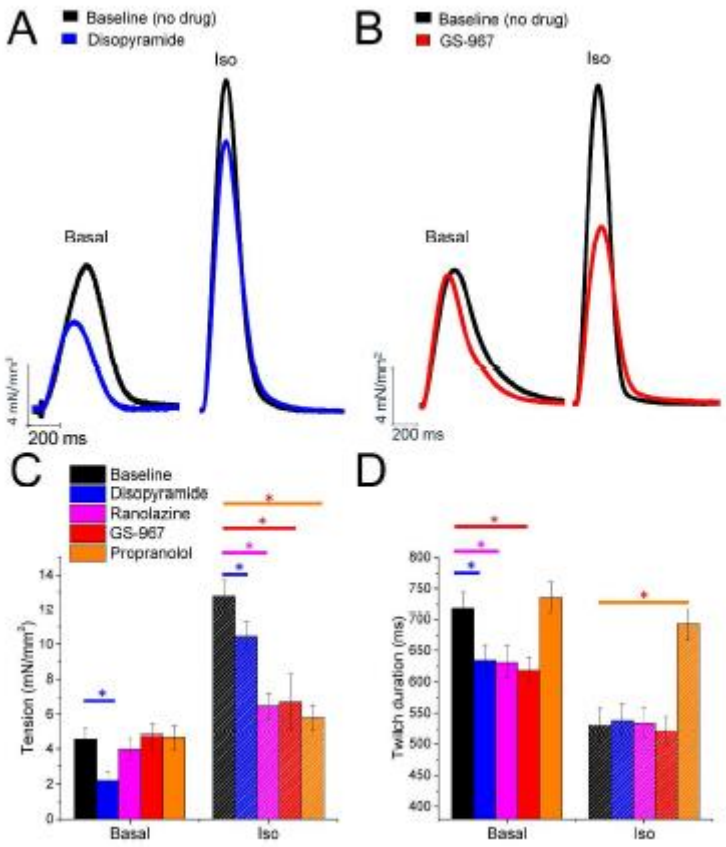

Figure 3 (next page): GS-967 and ranolazine reduce contractile force only during adrenergic stimulation in HCM myocardium. (A) Representative superimposed force twitches elicited at 1Hz in HCM trabeculae in the absence (black trace) and presence (blue trace) of Disopyramide 5μM, at basal conditions (left) and in the presence of Isoproterenol 10^{-7}M (Iso). **(B)** Representative superimposed force twitches elicited at 1Hz in HCM trabeculae in the absence (black trace) and presence (blue trace) of GS-967 0.5μM, at basal conditions (left) and in the presence of Iso. **(C)** Amplitude of force twitches elicited at 1Hz at baseline (black), in the presence of Disopyramide (blue), ranolazine (magenta), GS-967 (red) or Propranolol (orange), at basal conditions (left) or in the presence of Iso (right). (D) Duration of force twitches (from stimulus to 90% repolarization) elicited at 1Hz at baseline (black), in the presence of Disopyramide (blue), ranolazine (magenta), GS-967 (red) or Propranolol (orange), at basal conditions (left) or in the presence of Iso (right). (**C-D**) Means ± standard error from 10 trabeculae from 7 HCM patients. *=P<0.05, linear mixed models corrected for paired comparisons.

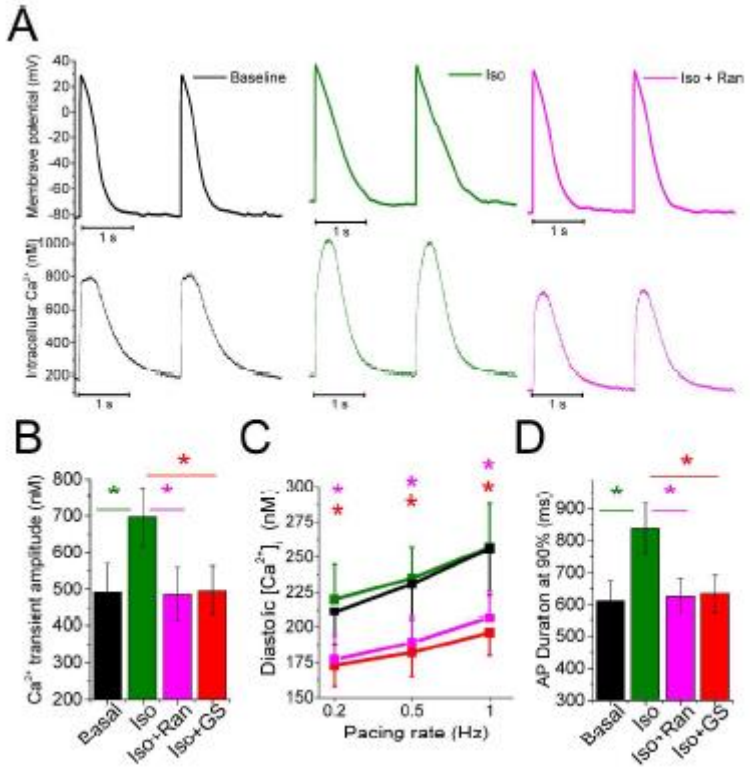

Figure 4: (A) Representative simultaneously recorded Action potentials (above) and Ca-transients (below) at baseline (left, black traces), in the presence of Isoproterenol 10^{-7}M (Iso, center, green traces) and in the presence of Ran 10µM added on top of Iso (right, magenta traces), elicited at 0.5 Hz in a HCM cardiomyocyte. **(B)** Ca-transient amplitude (0.5 Hz) at basal conditions (black), in the presence of Iso (green), in the presence of ranolazine 10µM added on top of Iso (Iso + Ran, magenta) or with GS-967 0.5µM added on top of Iso (Iso + GS, red). **(C)** Diastolic intracellular Ca-concentration ($[Ca^{2+}]_i$) at basal conditions (black), in the presence of Iso (green), in the presence of ranolazine 10µM added on top of Iso (magenta) or with GS-967 added on top of Iso (red), during regular stimulation at 0.2, 0.5 and 1Hz (steady state). **(D)** Action potential duration at 90% repolarization (0.5 Hz) at baseline (black), with Iso (green), with Iso + Ran (magenta) or with Iso + GS (red). **(B-D)** Means ± standard error from 21 cardiomyocytes from 7 HCM patients. *=P<0.05, linear mixed models, corrected for paired data.

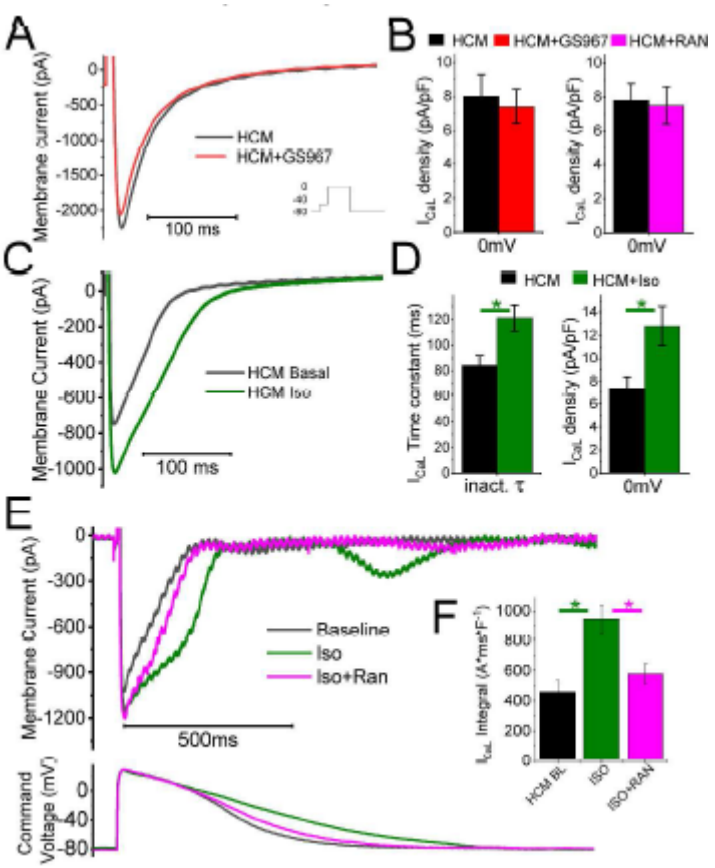

Figure5: Ranolazine and GS967 reduce total Ca^{2+} entry through L-Type Ca^{2+} -current under β-adrenergic stimulation. (A) Representative superimposed L-Type Ca-current traces at baseline (black trace) and in the presence of GS-967 0.5µM (red trace) from a HCM cardiomyocyte (the pre-pulse at -40mV is omitted from the traces). **(B)** L-Type Ca-current density at baseline (black), in the presence of GS-967 0.5µM (red) or 10µM ranolazine(magenta). Means ± standard error from 15 HCM cardiomyocytes from 5 patients. **(C)** Representative superimposed L-Type Ca-current traces at baseline (black traces) and in the presence of Iso (green) **(D)** L-Type Ca-current inactivation time-constant(left) and density (right) at baseline (black) and with Iso (green) in HCM cells (13 myocytes, 5 patients). **(E)** Representative L-type Ca-current traces recorded during AP-clamp at baseline (black), with Iso (green) and Iso+Ran (magenta); command voltage is shown below. **(F)** Integral of Ca-current during AP-clamp. Means ± standard error from 14 cells (5 patients). *=P<0.05, linear mixed models, corrected for paired data.

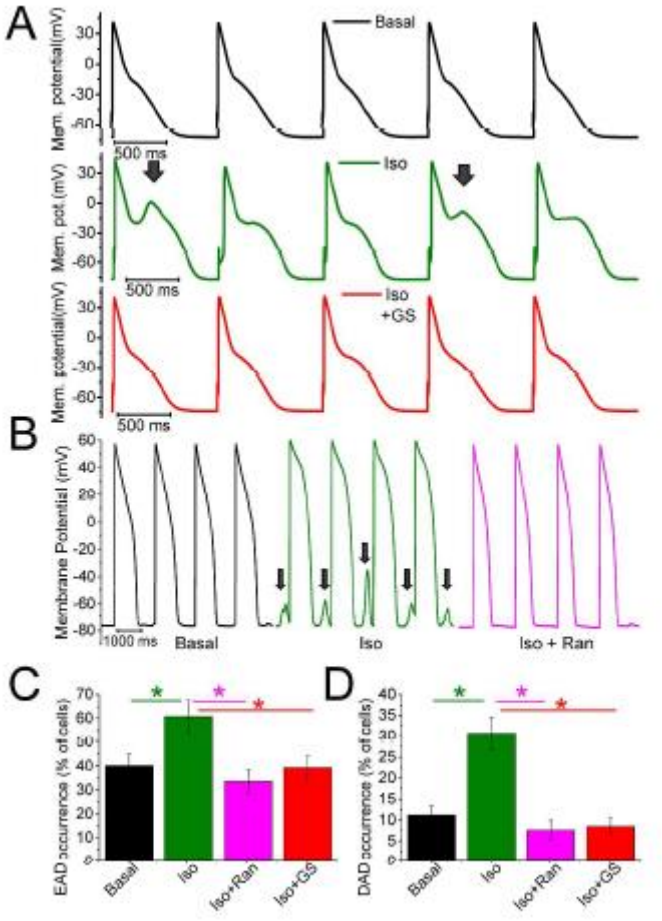

Figure 6: (A) Representative action traces at baseline (black traces), in the presence of Isoproterenol 10^{-7} M (Iso, green trace) and in the presence of GS-967 0.5 µM added on top of Iso (red traces), elicited at 1 Hz pacing rate. Ranolazine suppresses early after-depolarizations (marked by black arrows) that occur under Isoproterenol **(B)** Representative action potential traces at baseline, with Iso and Iso+Ran, 0.5Hz pacing rate. Ranolazine suppresses delayed after-depolarizations (marked by black arrows) that occur under Isoproterenol. **(C-D)** Percentage of HCM cardiomyocytes showing at least 2 early after-depolarizations (EADs, in **C**) or 2 delayed after-depolarizations (DADs, in **D**) during 3 minutes of continuous stimulation, at baseline (black), in the presence of Isoprotenerol (Iso), when ranolazine is added on top of Iso (magenta) and in the presence of GS-967 0.5µM added on top of Iso (red). Means ± standard error from 31 HCM cardiomyocytes from 9 HCM patients. *=P<0.05, linear mixed models corrected for paired comparisons.

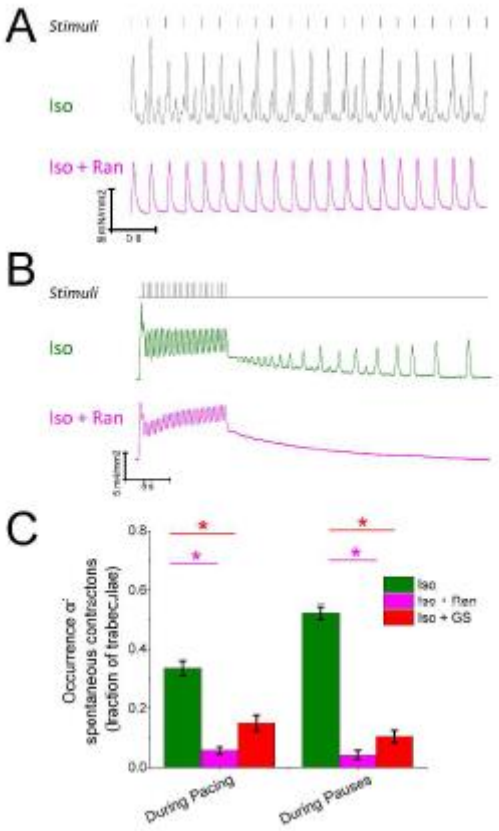

Figure 7: (A) Representative force traces at baseline (black traces), in the presence of Isoproterenol 10^{-7} M (Iso, green trace) and in the presence of Ranolazine 10 µM added on top of Iso (magenta traces), elicited at 0.5 Hz pacing rate. Ranolazine suppresses the spontaneous activity that occur under Isoproterenol **(B)** Representative force traces at baseline (black traces), in the presence of Isoproterenol 10^{-7} M (Iso, green trace) and in the presence of Ranolazine 10 µM added on top of Iso (red traces). After 10 second of burst pacing at 3 Hz, the stimulation was abruptly interrupted to induce spontaneous activity. (C**)** Means ± standard error from 15 HCM trabeculae from 12 HCM patients. *=P<0.05, linear mixed models, corrected for paired data.

Table 1: HCM Patients Characteristics

HCM Patients (n=22)	
Age at surgery	51 ± 8 yrs
Gender	Female 11/22 (50%)
NYHA Class II	13/22 (59%)
NYHA Class III	9/22 (41%)
Genotype	15/21 (7 MYBPC, 4 MYH, 4 SARCOMERE-NEG.)
Arrhythmic risk	
Syncope	7/22 (32%)
Non-sustained ventric. tachycardia	11/22 (50%)
History of Atrial Fibrillation	10/22(45%)
ICD implanted	3/22 (13%)
Echo features	
Maximal septal thickness	27±6 mm
Ejection fraction	66 ± 6 %
LVOT grad. at rest >30mmHg	22/22 (100%)
LVOT gradient at rest	81 ± 9 mmHg
LA end-systolic volume	101 ± 37 mL
Severe diastolic dysfunction (pseudonormalized,restrictive)	10/22 (45%)
Pharmacological Therapy	
Beta blockers	22/22 (100%)
Disopyramide	11/22 (50%)
Amiodarone	4/22 (18%)
Diuretics/ACE-Inhibitors	11/22 (50%)

Table 1: Clinical features of the HCM patients enrolled in the study. Clinical data refers to visits performed less than 1 month before the myectomy operation. Categorical data is expressed as proportion of patients; continuous values are expressed as mean ± standard deviation. NYHA= New York Heart

Association; MYPBC= Myosin-Binding Protein C; MYH= Myosin Heavy Chain; ICD= Implantable Cardioverter Defibrillator; LVOT= Left-Ventricular Outflow Tract; LA= Left Atrium.

Table 2: Effects of ranolazine and GS-967 on action potentials and intracellular Ca^{2+} in the presence of Isoproterenol.

Kinetics 0.5Hz(ms)	Ca^{2+} transients				Action Potentials	
	TTP	50%	90%	TOT. T.	APD50	APD90
Baseline*	163±41	568±54	1010±71	1178±78	699±47	792±50
Iso 10^{-7}M*	315±58	445±58	904±61	1219±79	854±59	981±68
Iso + Ran#	207±29	435±41	903±53	1132±61	706±34	796±40
Iso + GS‡	209±28	432±38	894±56	1128±60	708±32	799±38
***P* (Basel. vs Iso)**	**<0.05**	**<0.05**	**<0.05**	n.s.	**<0.05**	**<0.05**
***P* (Iso vs Iso+Ran)**	**<0.05**	n.s.	n.s.	n.s.	**<0.05**	**<0.05**
***P* (Iso vs Iso+GS)**	**<0.05**	n.s.	n.s.	n.s.	**<0.05**	**<0.05**

Table 2: Effects of ranolazine and GS-967 on action potentials and intracellular Ca^{2+} in the presence of Isoproterenol. Data are expressed as means ± standard error of mean. *: data from 25 HCM cardiomyocytes isolated from 7 HCM patient samples; #: 13 cells, 6 pts.; ‡=12 cells, 5 pts. P values were calculated using linear mixed models corrected for paired comparisons. TTP= time from stimulus to peak; 50%= time from peak to 50% decay of Ca-transients; 90%= time from peak to 90% decay of Ca-transients; TOT. T.= Total Ca^{2+}-transient duration (from stimulus to 95% decay); APD20= action potential duration at 20% of repolarization; APD50= action potential duration at 50% of repolarization; APD90= action potential duration at 90% of repolarization.